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**G (ALPHA) 12 IS REQUIRED FOR THROMBOXANE A₂ TO
REGULATE TUMOR CELL MOTILITY**

By

Babar Malik

M.B, B.S, Dow Medical College, PAKISTAN-2006

A thesis

Submitted in partial fulfillment of the requirements for the

Master of Science degree

Department of Molecular Biology, Microbiology and Biochemistry in the Graduate
School

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Thesis approval

G (ALPHA) 12 IS REQUIRED FOR THROMBOXANE A₂ TO
REGULATE TUMOR CELL MOTILITY

By

Babar Malik

A thesis

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Approved by:

Dr. Daotai Nie, chair

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Southern Illinois University Carbondale

24th June, 2011

AN ABSTRACT OF THE THESIS OF

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TITLE: G (ALPHA) 12 IS REQUIRED FOR THROMBOXANE A₂ TO REGULATE TUMOR CELL MOTILITY

MAJOR PROFESSOR: Dr. Daotai Nie

Thromboxane (TX) A₂ is a prostaglandin produced by metabolism of arachidonic acid through cyclooxygenases and thromboxane synthase. TXA₂ is biologically active, mainly through activating its cognate, seven transmembrane, G protein coupled receptor. It was previously reported that thromboxane A₂ receptor (TP) was expressed in prostate cancer, and further activation of this receptor elicited cell contraction and modulated tumor cell motility through regulating small GTPase RhoA[1]. This study aims to identify G alpha protein(s) involved in thromboxane A₂ signaling in tumor cell motility.

Methods: The expression of G alpha proteins in the PC3MM cells was studied by real time PCR. Cell contraction assay was performed by staining cells with TRITC phalloidin. Tumor cell motility and invasion were evaluated using wound healing assay and transwell Boyden Chamber assay. To determine the roles of G alpha protein in thromboxane A₂ signaling, we expressed different alleles of G alpha proteins (wild type, constitutive active) in PC3MM cells and then the subsequent effects on cell contractions was determined. We also depleted G alpha protein expression by short hairpin RNA and examined subsequent changes in cell contraction and migration after activation of TP. G (alpha) 12 has been reported to play critical role in cell proliferation in vitro and tumor growth in vivo. These findings were validated by performing BrdU Incorporation assay, Cell proliferation assay and cell cycle analysis. Quantitative analysis of the epithelial to

mesenchymal transition associated genes was performed by real time PCR. In vivo experiments were performed in order to validate in vitro findings.

Results: PC3 MM cell line had the endogenous level of all the G alpha protein (G alpha 12, G alpha i1, G alpha 11, G alpha 13), but not G alpha q. Overexpression of G alpha 12/13 increased cell contraction after activation of thromboxane A₂ receptor with U46619. Expression of constitutively active G alpha 12 by itself was sufficient to cause cell contraction, regardless whether TP is activated or not. We have identified two potent shRNAs that can silence the G Alpha 12. Depletion of G alpha 12 via shRNAs reduced cell contraction after TP activation. Tumor cells with depleted G alpha 12 had shown decreased tumor cell motility, invasiveness and cell proliferation. Cell cycle analysis showed that G alpha 12 depleted cells exhibit G1/G0 arrest. Quantitative expression of EMT associated genes was reduced in G alpha 12 depleted cells exhibiting increase in the E cadherin / Vimentin ratio. G alpha 12 depleted cells in comparison to scramble control show reduced rate of cell proliferation reminiscent of in vitro findings.

Conclusion: This study presents compelling evidence that G alpha 12 plays a major role in thromboxane A₂ regulation of prostate cancer invasion and metastasis. Silencing of G alpha 12 with shRNA may therefore provide a promising therapeutic strategy for prostate cancer patients.

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INTRODUCTION

Over the years prostate cancer has become the most common male malignancy and one of the leading cause of cancer death in American men[2]. Prostate cancer has been synonymous with low grade of replication potential along with old age. Median age for prostate cancer to develop is around 67 years (SEER Cancer Statistics). Overall there has been a decline in the rate of incidence of prostate cancer, it's not due to the fact that we have been able to find a cure for the cancer itself but due to better diagnostic tools or screening procedures [3]. Prostate cancer can be diagnosed with blood serum analysis for PSA, digital rectal exam and transurethral ultra-sonography (TRUS). Prostate cancer substantiates itself with an alteration of Prostate Specific Antigen (PSA) level in the blood and urinary dysfunction, along with impact on sexual function and performance. The current modalities for treatments include surgery, radiation, and adjuvant hormonal therapy. Although these therapies are relatively effective, majority of patients diagnosed with localized prostate cancer ultimately recidivate. Therefore, the potential risk confronted by prostate cancer patients is the development of metastasis, which presents with additional symptoms, such as bone pain, weakness in legs, and urinary and fecal incontinence [3]. Immense efforts are underway to develop therapeutics for the treatment of prostate cancer metastasis. Epidemiologic studies have shown that individuals who take nonsteroidal anti-inflammatory drugs on a regular basis have a 40–50% reduction in mortality from colorectal cancer [4]. One trait common in all of the non steroidal anti-inflammatory drugs is their ability to inhibit cyclooxygenase (COX), an enzyme required in the conversion of arachidonic acid to prostaglandins[5]. COX possesses two isoforms, COX-1 and COX-2[6]. COX-1 and 2 is expressed at high levels in both bladder and

prostate tumors in humans [7], indicating a potential roles for COX in cancer progression. Invasiveness of the prostate cancer was reduced by treatment with sulindac sulfone, a known COX inhibitor [8]. COX-1 is a constitutively expressed enzyme that generates prostaglandins (PG) and thromboxanes from arachidonic acid [5]. Thromboxane synthase which originates from arachidonic acid is being known as potent mediator of cancer cells metastasis [9]. TXA₂ stimulates platelet aggregation which is arbitrated by the activation of glycoprotein complex IIb/IIIa[10].TXA₂ is readily hydrolyzed to the biologically inactive form TXB₂ in aqueous solution[11]. Due to its unstable nature, TXA₂ primarily functions in an autocrine or paracrine manner within vicinity of production [12]. It has been shown that low dose of acetylsalicylic acid (asprin) which impedes the formation of TXA₂ by irreversibly inhibiting cyclooxygenase-1 (COX-1), exhibits 30% reduction in colorectal cancer[13] and possibly other cancers and esophageal cancer[4, 14, 15] including prostate cancer[13].

TXA₂ is biologically active, mainly through its cognate, seven transmembrane, G protein coupled receptor [16]. Ligand binding to the G-protein-coupled receptors (GPCRs) transmit signals through conformational changes causing interaction with heterotrimeric GTP-binding proteins (G proteins), which in turn activates the downstream signaling cascades. After the activation of the receptor these signals are being further carried forward by the heterotrimeric G proteins, which are adhered to the inside surface of the cell membrane. Heterotrimeric G proteins consist of α , β and γ subunits. Upon activation of the G protein coupled receptor it induces a conformational change which manifest itself by acting as a guanine nucleotide exchange factor that exchanges GDP for GTP on the G alpha subunit, this action initiates the dissociation of G alpha from G $\beta\gamma$ dimer and

its receptor[17] . It is known that thromboxane A₂ receptor (TP) which binds to its seven transmembrane G protein coupled receptor is expressed in prostate cancer, and further activation of this receptor elicit cell contraction and modulated tumor cell motility through regulating small GTPase RhoA[1].

Based on these findings we hypothesized that G alpha subunits functions as upstream regulators of GTPase form of Rho A activity by responding to extracellular stimuli which manifest as modulating prostate cancer cell motility and cytoskeleton reorganization. G alpha subunit is divisible into four families: G alphas, G alpha i/o, G alpha q/11and G alpha12/13. G alpha12 and Galpha13, are the ones which are activated by various stimuli, such as thromboxane A₂, lysophosphatidic acid (LPA), and thyroid-stimulating hormone receptors[18-22] . G alpha 12 and G alpha 13 are known to modulate variety of cellular processes, such as actin cytoskeleton reorganization, neurite retraction, platelet aggregation, and apoptosis[19, 21, 23, 24]. In spite of the fact that G alpha12 and G alpha 13 perform functions that coincide with each other however they seem to distinguish in their abilities to conjoin to different ligands for different physiological effectors[18, 21, 25]. Microarray analysis shows that G alpha12 is highly expressed in nasopharyngeal carcinoma [26] and it modulates the actin cytoskeleton reorganization which promotes highly invasive nature of these cancer[26, 27].G alpha 12 stimulates multiple signaling pathways by the activation of the small GTPase Rho via specific Rho guanine nucleotide exchange factors (RhoGEFs)[27]. G alpha 12 stimulate Rho activity through LARG and PDZ-RhoGEF[27].p115RhoGEF was the first RhoGEF to be recognized as a connection between G alpha 12/G alpha13 and Rho activation[28]. In small cell lung carcinoma, G alpha 12 and 13 have been known to promote invasiveness [29]. The ectopic expression

of wild-type or an activated mutant of G alpha 12 causes cancerous transformation of fibroblast cell lines such as NIH-3T3 cells [30]. Multitude of studies shows that G alpha12 and G α 13 plays an important role in terms of defining the downstream signaling cascade but there role with relation to thromboxane A₂ receptor activation was still ambiguous in prostate cancer cells. Since it has been proposed that gene modulation is one of the most sought after strategy since genomic instability leads to progression of cancer which later could result in its metastasis, so knockout experiments were being carried out which revealed that G alpha 13 depleted cells resulted in impaired angiogenesis and intrauterine death[31]. Knockdown of G alpha 12 has been reported to strongly activate the basal ERK, sustained activation of ERK results in DNA damage induced apoptosis [32]. These studies reflect that G alpha 12 and G alpha 13 might play a pertinent role in cellular signaling pathway in prostate cancer cells. Various methods can be employed to inhibit a gene expression, so far small hairpin RNA (shRNA) represents a considerable promise for cancer therapy due to its potent and specific knockdown effect[33]. In the present study, we intend to silence the G alpha 12 expression in prostate cancer cells using synthetic shRNA and evaluate its effects on tumor cell invasiveness and growth by employing both in vivo and in vitro experiments.

MATERIALS AND METHODS

Materials

QS transfection reagent was provided by Dr Yin Yuan Mo. TRIzol reagent, fetal bovine serum (FBS), TRITC-phalloidin stain and streptomycin were purchased from Invitrogen (Carlsbad, CA). U46619 which is a structural homolog of Thromboxane A₂ was purchased from Cayman Chemicals (Ann Arbor, MI). Cell culture medium RPMI was purchased from Lonza Bio-whittaker (Walkersville, MD). Dulbecco's Phosphate-Buffered Saline (DPBS) and penicillin was obtained from Cellgro (Manassas, VA). Puromycin was obtained from Clontech, Inc (Mountain view, CA). Wild type and constitutively active form of G alpha subunits (G alpha 12, 13, q, 11 and i1) were purchased from Missouri S&T cDNA Resource Center (Rolla, MS). Blocking buffer was purchased from Licor Biosciences (Lincoln, Nebraska). SYBR Green-1 dye universal master mix and reverse transcriptase were purchased from Takara Biotechnology, Inc. (Otsu, Shiga, Japan). Reagents for reverse transcriptase polymerase chain reaction were purchased from Thermo Fischer scientific (Barrington, IL). Falcon Culture cell culture inserts for 24 well plates with 8.0 μm pore Translucent PET Membrane were purchased from BD Biosciences (Mississauga, Ontario). β-actin antibody was purchased from Sigma-Aldrich Inc. (St Louis, MO), and the G alpha 12 polyclone antibody was obtained from Santa Cruz Biotech. (Santa Cruz, CA). BrdU cell proliferation assay kit was purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture

Human prostate cancer cell lines (PC-3MM) were obtained from Dr Kounosuke Watabe. PC-3MM cells were cultured in RPMI 1640 with 10% FBS and streptomycin (100 $\mu\text{g}/\text{mL}$). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. The culture medium was replaced every 2–3 days, and the cells were treated with PBS in order to lift them up when they reached 80~90% confluence.

Transfection of wild type and constitutively active G alpha subunits

PC-3MM Cells were transfected with wild type and constitutively active form of G alpha subunits (G alpha i1, q, 12, 13 and 11) using QS transfection reagent. Cells were seeded in a 6-well plates at a density of 0.5×10^5 cells/ well in RPMI 1640 medium containing 10% FBS and streptomycin (100 $\mu\text{g}/\text{mL}$). 1 μg of G alpha subunit was mixed with 2 μl QS transfection reagent along with 100 μl of FBS free medium RPMI1640, it was incubated at room temperature for 15 min to form a complex. The cell culture medium was then removed, and 103 μl of $G\alpha/\text{QS}$ transfection complex was added drop wise to the cells. After twenty-four hours, transfection medium was replaced with fresh RPMI-1640 medium containing 10% FBS, and streptomycin.

G alpha 12 shRNA Constructs

Two predesigned commercially available shRNAs (shRNA 909 and shRNA911) were purchased from Origene, Inc (Rockville, MD). These shRNAs are of 29 nucleotides each one having a sequence as shRNA909 (TGGTGGAGTCCATGAACATCTTCGAGACC) and shRNA911 (CTGCTGGAGTTCCGCGACACCATCTTCGA). Both of these

predesigned shRNA sequences were verified against the human genome database to annihilate cross linking with other-target genes. Scrambled shRNA was purchased from Origene, Inc and it was used as a negative control.

Transfection of G alpha12 shRNA

PC-3MM Cells were transfected with shRNA using QS transfection reagent. Cells were seeded in a 6-well plates at a density of 0.5×10^5 cells/ well in RPMI 1640 medium containing 10% FBS and penicillin/streptomycin (100 $\mu\text{g}/\text{mL}$). After 24 hours, cells were transfected with shRNA using QS transfection reagent. 1 μg of shRNA was mixed with 2 μl QS transfection reagent along with 100 μl of FBS free medium RPMI 1640; it was incubated at room temperature for 15 min to form a complex. The cell culture medium was then removed, and 103 μl of shRNA/QS transfection complex was added drop wise to the cells. After twenty-four hours, transfection medium was replaced with fresh RPMI-1640 medium containing 10% FBS, and streptomycin.

Real-Time RT-PCR

Total RNA was isolated from cell pellets using TRIzol reagent (Invitrogen) as per the manufacturer's protocol. RNA (1 μg) was converted to cDNA using dNTP, primers, and Taq polymerase Reverse Transcriptase Reagent. One microgram of cDNA was amplified by Real-Time PCR using the MJ Mini Thermal Cycler (Biorad Life Sciences, Hercules, CA). GAPDH was used as a loading control. The primers used for G alpha subunits are as follows

G alpha q 5'-TGGGTCAGGATACTCTGATGAAG-3'(forward) and 5'-TGTGCATGAGCCTTATTGTGC-3' (reverse)

G alpha i1 5'-GCTCAACCAAATTACATCCCGA-3'(forward) and 5'-TCGTAGTCACTCAGTGCTACAC-3 (reverse)

G alpha 11 5'-CCAAGCTCGTCTACCAGAACA-3'(forward) and 5'-TGACGTACTGATGCTCGAAGG-3 (reverse)

G alpha 13 5'-TCCAAGGAGATCGACAAATGC-3'(forward) and 5'-CCAGCACCCCTCATACCTTTGA-3 (reverse)

G alpha 12 5'-AAGGGCTCAAGGGTTCTTGTT-3'(forward) and 5'-TGATGCCAGAATCCCTCCAGA-3 (reverse).

To confirm the knockdown of G alpha 12 we performed quantitative PCR, 1 microgram of cDNA was amplified using SYBR Green Master mix on 7500 Real Time PCR System by Applied Biosciences (Foster City, CA). PCR products were subjected to a melting-curve analysis. Comparative threshold (Ct) method was used to calculate the relative amount of mRNA of treated sample in comparison to control samples. Each sample was performed in triplicate, and the mean value was calculated.

Cell Contraction Assay

PC-3MM cells (10,000 cells/well) were plated at low density in 6 well plate and incubated at 37° C in a humidified environment having 5% of CO₂. Cells were placed under serum free condition for 24 hours. Replace the media and put 300nMol U46619 (structural analog of TXA₂) for 15 min. Removed the media and add 2% paraformaldehyde for 15 min. Wash the cells with PBS for 15 min. Treated the cells with 0.1x of triton.

Removed triton and washed the cells with PBS for 1 min. Treated the cells with TRITC phalloidin stain for 15 min, remove the staining media and wash the cells with PBS for 15 min. Images were taken using an inverted microscope.

Western Blot Analysis

PC-3MM cells transfected with the G alpha 12 shRNA for 24 h, 48 h post transfection, protein was isolated using 2x SDS buffer which contains protease and phosphatase inhibitor. Same amount of total protein was used for all the samples and was separated on a SDS PAGE gel (percentage of gel depended on the molecular weight of the protein). The separated proteins were transferred to a nitrocellulose membrane, blocked, and probed with appropriate antibodies. The protein bands were then visualized using secondary antibodies. The membranes were later scanned with Odyssey infrared analyzer.

Wound-Healing Assay

PC-3MM cells were seeded in 6-well plates, Once the cells reached 90% confluency, using a (yellow) pipette tip make a straight scratch which simulates a wound by keeping the tip under an angle of around 30 degrees to keep the scratch width limited. The detached cells were removed by washing with PBS. Then, the cells were incubated in the incubator supplemented with 5% CO₂ at 37°C. The wound-healing process was monitored microscopically over the period of time as the cells moved into the wound area, and the images were taken from the same place at various intervals using an inverted microscope.

Cell Migration Assay

The effect of shRNA on invasiveness and metastatic potential of prostate cancer cells was evaluated using Boyden Chamber assay. Twenty-four hours after seeding the PC-3MM cells. They were treated with PBS in order to lift them up and later it was re-suspended in FBS-free RPMI- 1640 medium. A total of 5×10^3 PC-3MM cells were suspended in 200 μ l volume of serum free RPMI 1640 were plated in the top chamber with an un-coated membrane (0.3cm² growth surface, 8.0 μ m pore size, Polyethylene terephthalate) for migration assay. 10% FBS containing RPMI1640 was added in the lower chamber as a chemo-attractant. After incubation for 24 h, the cells migrated to the lower surface of the membrane. The cells that did not migrate through the pores were mechanically removed with a cotton swab. After 18 hours of treatment, images of migrated cells were obtained by an inverted microscope at a magnification of 200x.

BrDU Incorporation Assay

PC-3MM cells (10,000 cells/well) were plated in 96 well plate and incubated at 37° C in a humidified environment having 5% of CO₂. BrdU solution was prepared by diluting the 1000X stock in cell culture medium at 1:100 dilutions. After 24 hours, remove media that contains BrdU solution. After adding 100 μ l of the fixing solution incubate the plate at room temperature for 30 min. Remove solution and add 100 μ l/well of premade 1x detection antibody solution. Keep the plate at room temperature for 1 hour. Remove solution and wash plate with 1 x wash buffer. Now add 100 μ l/well prepared 1x HRP-conjugated solution and keep it at room temperature for 30 min. Remove the solution and wash plate with 1 x wash buffer. Add 100 μ l of TMB substrate. Incubate the plate for 30

min. Keep an eye on color change as it may be imperative to stop the reaction prior to development time of 30 min. Add 100µl STOP solution. Read absorbance at 450nm within 30 min for optimal results.

Cell Proliferation Assay

PC-3MM cells (100000 cells/well) were plated in triplicate at a low density in a 6-well plate and incubated for 24 hours in serum containing RPMI 1640 medium. The incubated cells were treated with 1 ml of PBS at 24, 48 and 72 hours in order to detach the cells for counting. The cells are being treated with trypan blue which stains the damaged cells and count viable cells that exclude the dye. The effect of G alpha 12 shRNA on cell proliferation was measured using Beckman Coulter Vi-cell Cell Viability Analyzer (GMI Inc. Minneapolis, MN).

MTS Assay

MTS assay is a colorimetric method for evaluating the number of viable cells in culture. The MTS compound is reduced by viable cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan is directly proportional to the number of living cells present in culture which can be recorded at 490nm of absorbance. PC-3MM cells (10000cells/well) were seeded in at low density in triplicate in a 96-well plate. The cells were incubated in a humidified environment in 5% CO₂ at 37° C. The incubated cells were treated with MTS solution after 48 hours. Absorbance was recorded at 490nm within 30 min for optimal readings. The effect of G alpha 12 shRNA

on cell proliferation was measured using Thermo Elektron Corporation (Multiskan Spectrum).

Cell Cycle Analysis Assay

PC-3 MM cells transfected with G alpha 12 shRNA were incubated at 37°C in a humid environment having 5% CO₂. When cells reached 80~85% confluency, they were detached by PBS, cells were collected and centrifuged at 1200 rpm at 4°C for 5 min, decant supernatant and gently re-suspend the cells in PBS. To fix the cells add 0.7ml in ice-cold 70% ethanol and leave it on ice for 1hour. After fixing, cells were washed with PBS once and then re-suspend cell pellet in 0.25ml of PBS, add 5µl of 10mg/ml Rnase A. Incubate it at 37 °C for 1 hour. Add 10 µl of 1mg/ml PI solution. Keep it under cover and at 4°C until analysis. Cell cycle analysis was carried out with FACS Flow cytometer (BD Biosciences) at 488nm. The result was confirmed from three independent experiments; each group had three samples.

Tumor cell lines & in vivo experiments

The PC-3MM cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin(100µg/ml).Animal use was according the guidelines set by Animal Welfare Act(AWA)/United States Department of Agriculture (USDA).For induction of tumors nude mice were injected subcutaneously with 2×10^6 scramble control as well G alpha 12 depleted PC-3MM cells at the back. The rate of tumor growth was monitored for 4 weeks.

Statistical Analysis

Student's t test was employed to examine the difference between two groups. Data was expressed as the mean \pm standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS

Endogenous expression of G alpha subunits in PC-3MM

The endogenous expression of G alpha subunits was analyzed by real time PCR. G alpha subunits were amplified from the cDNA which was synthesized from mRNA by reverse transcriptase PCR. As shown in fig 1, All cell lines tested had the endogenous expression of all the G alpha subunits (G alpha 12, G alpha i1, G alpha 11, G alpha 13 and G alpha q), with the exception that PC 3 and PC3 MM cell lines which lacked G alpha q as shown in Fig 1.

G alpha12 and G alpha 13 activation causes actin cytoskeletal reorganization

In order to determine the role of G alpha subunits in thromboxane A₂ signaling, we expressed different alleles of G alpha proteins (wild type, constitutive active) in PC3MM cells and then the subsequent effects on cell contraction were determined. The rationale behind transfecting the constitutively active form was to use it as a reference control since it would exhibit its effect even in the absence of the ligand. It was observed that overexpression of G alpha 12/13 increased cell contraction after activation of thromboxane A₂ receptor, when treated with U46619. Expression of constitutively active G alpha 12 (Fig 2A) or G alpha 13 (Fig 2B) was sufficient to cause cell contraction, regardless whether TP is activated or not. Cells that exhibited rounded morphology having the length twice less than its width were counted.

Inhibition of U46619 induced cell contraction by G alpha Q

Profiling of G alpha subunits revealed a loss of G alpha Q in PC-3 or PC-3MM cells. U46619 induces cell contraction in PC-3 or PC-3MM cells efficiently but not in DU145 cells, even though DU145 cells express receptors for thromboxane A2. We hypothesize that G alpha Q may attenuate TP signaling to cell contraction. To test this hypothesis, PC-3MM cells were transfected with expression constructs for G alpha Q or constitutively active G alpha Q and then treated with U46619. Cells were then fixed, stained, and scored for contraction. As shown in Figure 3, restoration of G alpha Q or expression of active G alpha Q blocked U46619 induced cell contraction. Untransfected cells were used as controls. The results suggest that G alpha Q subunit inhibits U46619-induced cell contraction.

Suppression of G alpha 12 with commercially available shRNAs

Two commercially prepared synthetic shRNAs that would target human G alpha 12 mRNA were transfected in PC-3 MM cells. Suppression of G alpha 12 was examined in PC-3MM cells by shRNAs at the mRNA level using real-time RT-PCR. A scrambled shRNA was used as a negative control. Compared to the scrambled shRNA, both shRNA 909 and shRNA 911 exhibited an efficacious suppression of G alpha 12. Furthermore, the suppression of G alpha 12 at the protein level was confirmed by western blot. As shown in Fig 4, both shRNA909 and shRNA911 dramatically depleted the G alpha 12 protein expression in PC-3MM cells, which is consistent with the suppression at the mRNA level. shRNAs constructs were further used for following biological studies in prostate cancer cells.

G Alpha 12 depleted cells inhibit actin cytoskeleton reorganization

Inhibition of G alpha 12 decreased cell contraction after activation of thromboxane A2 receptor with U46619. Cells were transfected with short hairpin RNA for G alpha 12, in the presence of a GFP marker construct pEGFP, and then serum starved for 24 hours. Cells were treated with U46619 and EtOH for 15 min. fixed and then stained with TRITC phalloidin staining. As shown in Fig 5, scramble control cells in comparison to stable knock down of G alpha 12 showed greater contracting ability. Contractions of cells, transfected or untransfected, were scored under an epifluorescence microscope.

Suppression of G alpha 12 Inhibits Cell Motility

We investigated the effect of G alpha 12 inhibition on cell motility and invasiveness of prostate cancer cells. Wound-healing assay is an in vitro assay which portrays the in vivo motility of cancer cells. When cells reached 90% of confluency, they were disrupted by scratching a line through the layer to simulate a wound in the cell monolayer. The rate of cell migration is measured by the amount of time required to fill the gap and it depicts the wound healing process. Cells having depleted G alpha 12 showed slower rate of migration as compared to the cells treated with scrambled shRNA when treated with U46619 (structural analog of TXA₂). After 8 hours, cells transfected with the scrambled shRNA had filled the gap about 75~80%, while the cells treated with the G alpha 12 shRNA still had an unhealed gap (Fig 6), indicating that depletion of G alpha 12 can efficiently suppress the motility of prostate cancer cells.

Depletion of G alpha 12 inhibits cell migration

We investigated the effects of G alpha 12 depletion on the migrating abilities of prostate cancer cells. Migration of metastatic tumor cells towards a chemo-attractant is trademark

of these cells, and it is considered to be an essential step for tumor invasion and metastasis. The migration ability of PC-3MM cells was analyzed using Boyden chamber assay, which is a model to imitate the in vivo metastatic process. As shown in Fig.7, the scramble control cells treated with U46619 shows enhanced capability for migration in comparison to the cells having a stable knockdown of G alpha 12. Stable knockdown of G alpha 12 restricted the migrating ability of PC-3MM cells to 15% and 12%, respectively.

Effect of G alpha 12 shRNA on Cell Proliferation

Having shown that suppression of G alpha 12 refrains the cancer cells from acquiring migratory property, we performed three assays to examine the effects of G alpha 12 depletion on the rate of proliferation of prostate cancer cells. It was observed that G alpha 12 depleted PC-3MM cells exhibited slower rate of growth and viabilities when compared to cells transfected with scrambled shRNA, These findings were consistent in all the three assays (MTS assay, BrdU incorporation and trypan blue cell proliferation assay) as shown in fig 8.

Effect of G alpha 12 depletion on Cell cycle

We performed cell cycle analysis to determine whether knockdown of G alpha 12 precede to changes in the cell cycle distribution of prostate cancer cells. Cells transfected with G alpha 12 shRNAs in comparison to scrambled shRNA treated cells effectuate a block in G1/G0 phase distribution. The percentages of cells in G1/G0 phase in cells having depleted G alpha 12 for both the clones (shRNA 909,shRNA 911) were 53% and

64% , whereas 44% having scrambled shRNA were in G1/G0 phase. This G1/G0 phase was accompanied by repression in G2/M and S phase distribution in both G alpha 12 depleted cells. This difference in G1/G0 phase is statistically significant ($p=0.05$). The results of cell cycle analysis are consistent with findings of the cell proliferation assay. These results demonstrated that G alpha 12 inhibition negatively regulates cell proliferation in prostate cancer as shown in Fig 9.

Effect of G alpha 12 inhibition on EMT associated genes

Whenever a metastasis takes place in primary cancer it is always preceded by a complimentary change which is called as epithelial to mesenchymal transition (EMT). EMT causes transient morphologic and biological changes in cells that will alter cell motility and contact with neighboring cells[34]. The knockdown of G alpha 12 in PC-3 MM causes increased expression of all those markers which causes increased cell-cell interaction such as E cadherin and claudin 1 along with concomitant decrease in the expression of the vimentin, snail, snail 2, beta catenin, MMP9, MMP2(matrix metalloprotease), Zeb 1(Zinc finger E box binding homeobox binding 1), fn 1(fibronectin), fsp 1(fibroblast secretory protein) and sip 1 (Fig 10). These results were further verified by western blot. Taken together these results reflect that inhibition of G alpha 12 reduces EMT.

Effects of G alpha 12 suppression on tumor growth and progression

In order to confirm our in vitro findings we carried out in vivo experiments in nude mice. 2×10^6 cells having scramble control and G alpha 12 depleted cells were injected

subcutaneously in mice. The rate of growth of tumor was monitored for a period of 4 weeks. As shown in fig 11, scramble control cells showed robust rate of tumor growth as compared to the depleted G alpha 12 cells. These findings were consistent with our in vitro findings.

DISCUSSION

It is known that in order to metastasize, cancer cells need to acquire motility. During cell migration, a cell contracts at the trailing edges. Cytoskeleton reorganization is also required for the the formation of lamellipodia and fillopodia (cytoskeleton actin protein)[35]. Rho A is a small GTPase regulated protein known to control the actin cytoskeleton structure which results in the formation of stress fibers. Rho A modulates the actin cytoskeleton reorganization by activating ROCK which ends up activating cofilin through LIM kinase activation [36].

It has been shown that TXA_2 by binding to its cognate G protein coupled receptor causes the activation of RhoA which goes on to bring about actin cytoskeleton reorganization and manifests in the form of cell motility[1]. Since G protein coupled receptors channelize there activation through the heterotrimeric G proteins so we proposed that G alpha subunits might be the one regulating the downstream signaling of Rho A.

Principally there are four different G alpha subunits which can regulate the downstream signaling cascade such as G alpha s, G alpha i/o, G alpha q/11 and G alpha 12/13. We were not sure as to which subunit is regulating the activation of RhoA when TXA_2 ligand binds to its receptor.

In this study we explored that activation of the G alpha subunit is a critical step in the activation of Rho A and G alpha 12 is an important upstream regulator of the Rho A activation. G alpha 12 has been identified to play a major role in the metastasis of breast and nasopharyngeal cancers[26], though there role was still undeterminate in prostate cancer in relation to activation through TXA_2 .

In this study, we demonstrated that G alpha 12 shRNA even in the presence of U46619 (structural analog of TXA_2) significantly decreases invasiveness of prostate cancer cells.

We examined the effect of G alpha 12 depletion by two predesigned shRNAs in prostate cancer cells which was verified using real time RT-PCR and western blot analysis. Two commercially prepared potent shRNAs, shRNA- 909 and shRNA-911 were utilized to investigate their biological effects on prostate cancer cells. It is generally believed that G alpha 12/13 promotes the invasiveness as well as cell proliferation human small cell lung carcinoma in vitro and tumor growth in vivo[29]. We then analyzed the effect of G alpha 12 silencing on the invasive properties of prostate cancer by conducting wound-healing assay and chamber migration assay. Our studies showed that silencing of G alpha 12 significantly inhibit the would-healing process as well as cell migrating ability of prostate cancer cells as shown in Fig.4. The ability of the cells to close the gap with subsequent establishment of new cell-cell contacts depicts the migratory capability of cancer cells in metastasis. We further evaluated the migratory ability of prostate cancer cells having depleted G alpha 12 using a Boyden chamber assay. As shown in Fig6, G alpha 12 shRNAs significantly inhibited the migration capability of PC-3MM cells. These results are in full agreement with a recent finding that G alpha 12 promotes prostate cancer metastasis [27].

Kumar and colleagues reported the role of G alpha 12 in soft tissue sarcomas as to be a putative causative oncogene and over expression of wild type or GTPase deficient mutant of G alpha 12 causes oncogenic transformation of NIH3T3 cells[37]. This oncogenic transformation in NIH3T3 cells manifests by increased rate of cell proliferation, anchorage independent growth, decreased growth factor dependency and cytoskeletal changes [34]. They showed that suppression of PDGFR alpha and JAK3 rarefied the mutant G alpha 12 activity [34]. So they proposed that PDGFR alpha and

JAK3 provide an autocrine/paracrine loop for activation in G alpha 12 mediated cell proliferation. Based on these findings we performed three assays (MTS assay, BrdU Incorporation assay and Trypan blue cell proliferation assay) to depict the effect of G alpha 12 inhibition on the rate of cell proliferation in PC-3MM cells. As expected scramble control cells showed greater rate of cell proliferation as compared to the cells having a stable knockdown of G alpha 12. This result is in conformity with a report that knockdown of PDGFR alpha and JAK3 attenuated G alpha 12 and affected the rate of cell proliferation [3]. To further confirm these results, we performed cell cycle analysis. We observed that cells having a stable knockdown of G alpha 12 in comparison to scramble control showed slightly high distribution in G1/G0 phase, with a concomitant decrease in the cell population in S and G2/M phase. The difference is statistically significant from the control group. These results suggest that G alpha 12 shRNA has a significant effect on cell proliferation of PC-3MM cells.

Recently it has been reported that invasive phenotype is necessary for cells to cause bone metastasis[38]. Epithelial to mesenchymal transformation(EMT) in prostate cancer cells plays a pertinent role in not only prostate cancer maintenance but also bone metastasis and it is functionally associated with prostate cancer stem cells[38]. So we performed a quantitative analysis for the EMT associated genes, results show that G alpha 12 depleted cells in comparison to scramble control cells showed higher expression of those genes which modulates the cell- cell interaction (e cadherin and claudin) as a consequence it increased the E cadherin/vimentin ratio hence trying to restrict cells from acquiring mesenchymal trait which modulates the metastatic potential of prostate cancer cell line. We performed in vivo experiments in order to validate our in vitro findings. In vivo

studies carried out suggest that silencing of G alpha 12 and 13 leads to reduced tumor growth in comparison to control cells in Small cell lung carcinoma (SCLC) [29]. Results acquired after 4 weeks reflect that mice injected with knockdown clones of G alpha 12 showed slower growth rate in comparison to control cells.

In summary, results from this study provided significant evidence that G alpha 12 plays a pivotal role in the metastasis of prostate cancer cells. Considering the fact that the major risk faced by prostate cancer patients is the development of metastasis [39], the G alpha 12 shRNA may provide a very promising approach for prostate cancer therapy.

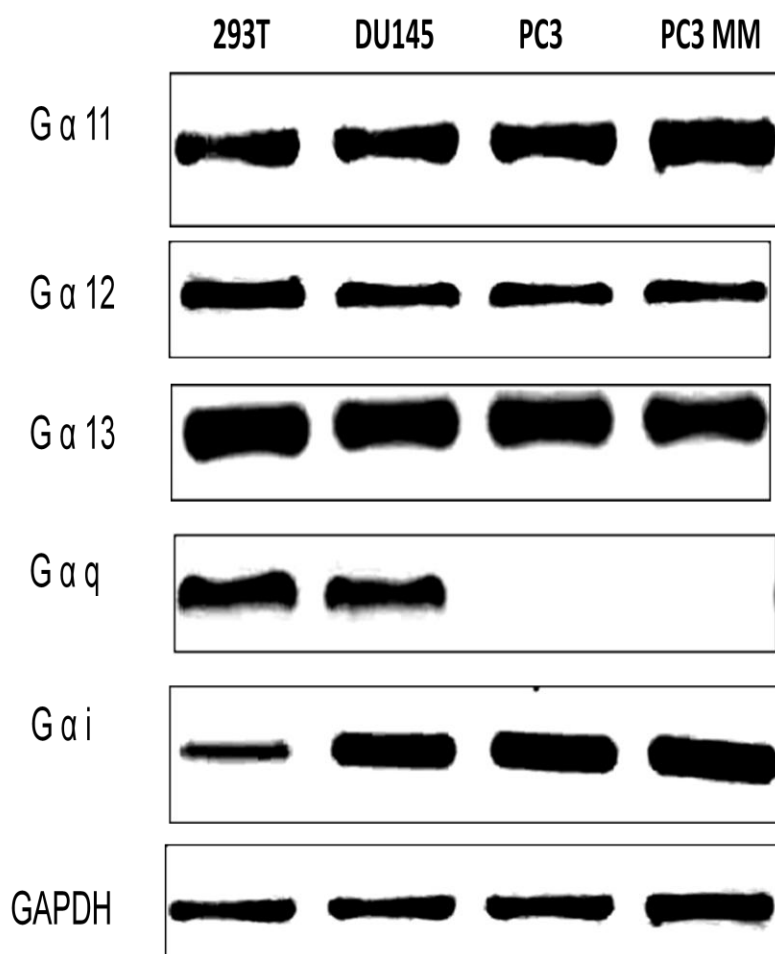


Fig 1. Expression profile of different G alpha sub-unit in benign and malignant cell lines. G alpha subunits were amplified from the cDNA generated by reverse transcriptase from mRNA. GAPDH was used as a loading control.

A)

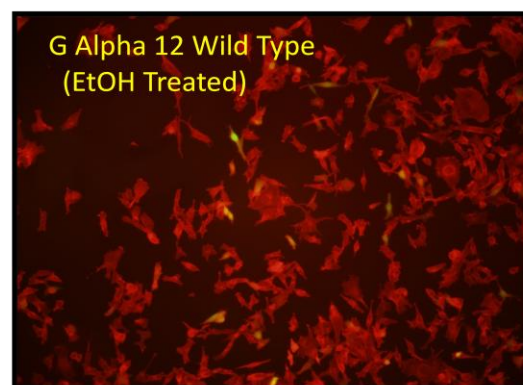
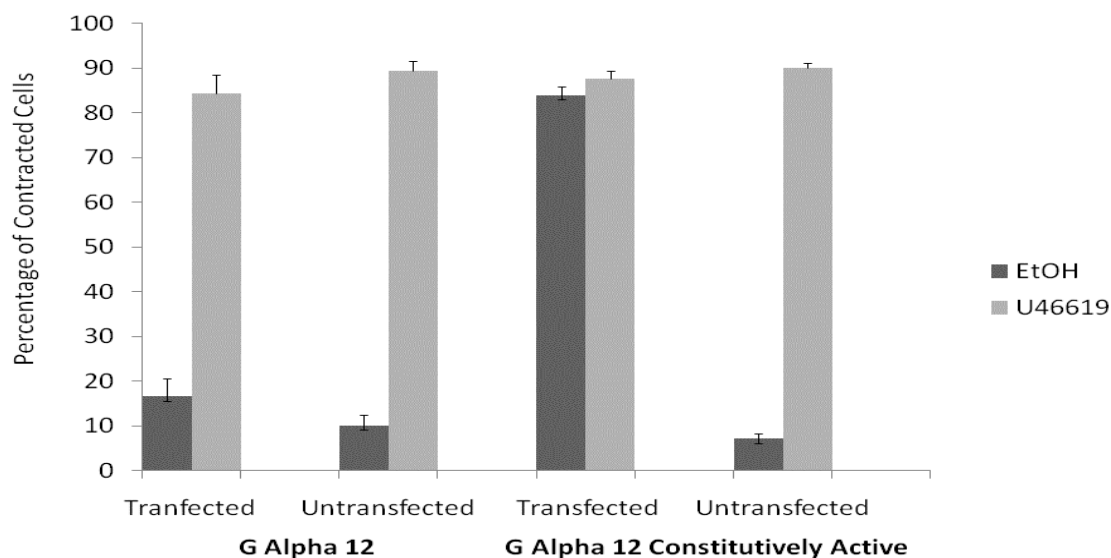


Fig 2. Overexpression of G alpha 12 (A) increased cell contraction after activation of thromboxane A2 receptor with U46619. Cells were transfected with expression vector for G alpha 12/13 or active G alpha 12/13, in the presence of a GFP marker construct pEGFP, and then serum starved for 24 hours. Cells were treated with U46619 and EtOH for 15 min. fixed and then stained with TRITC phalloidin staining. Contractions of cells, transfected or untransfected, were scored under an epifluorescence microscope.

B)

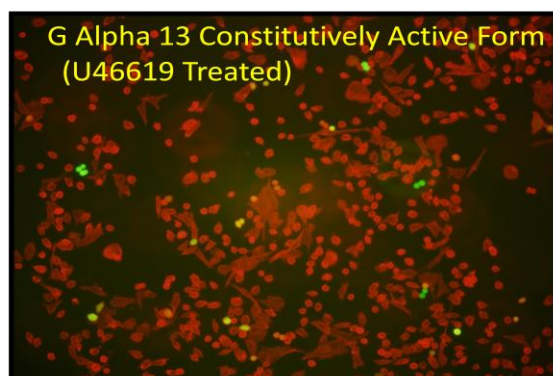
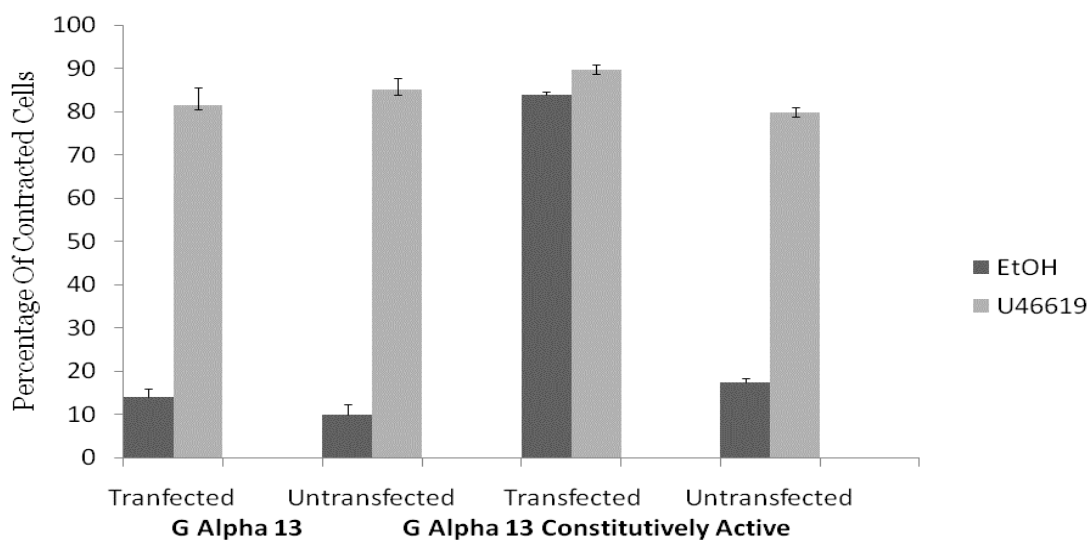


Fig 2. Overexpression of G alpha 13 (B) increased cell contraction after activation of thromboxane A2 receptor with U46619. Cells were transfected with expression vector for G alpha 12/13 or active G alpha 12/13, in the presence of a GFP marker construct pEGFP, and then serum starved for 24 hours. Cells were treated with U46619 and EtOH for 15 min. fixed and then stained with TRITC phalloidin staining. Contractions of cells, transfected or untransfected. were scored under an epifluorescence microscope.

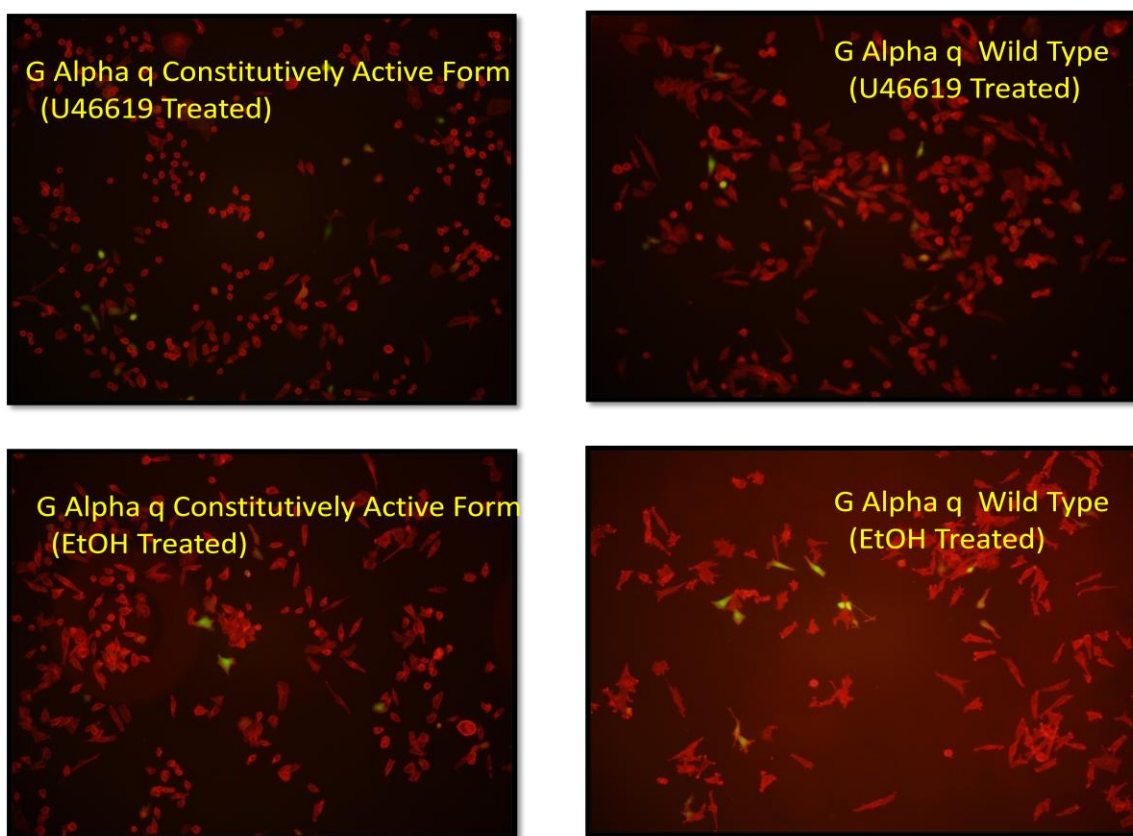
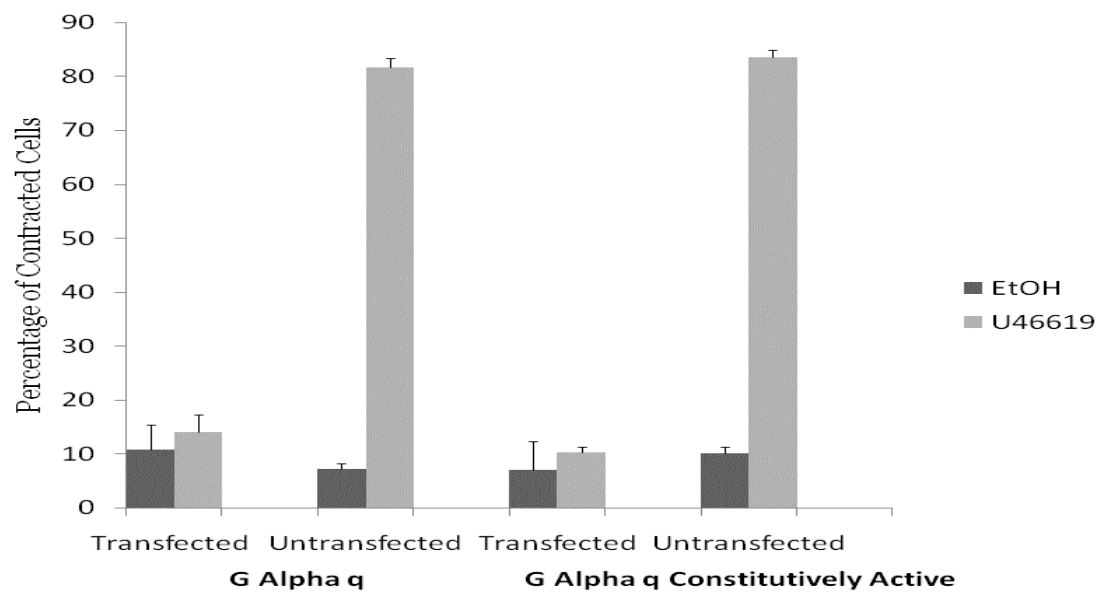


Fig 3. Suppression of U46619-induced contraction by G alpha Q. Cells were transfected with expression vector for G alpha Q or active G alpha Q, in the presence of a GFP marker construct pEGFP, and then serum starved for 24 hours. Cells were treated with U46619 and EtOH for 15 min. fixed and then stained with TRITC phalloidin staining. Contractions of cells, transfected or untransfected, were scored under an epifluorescence microscope.

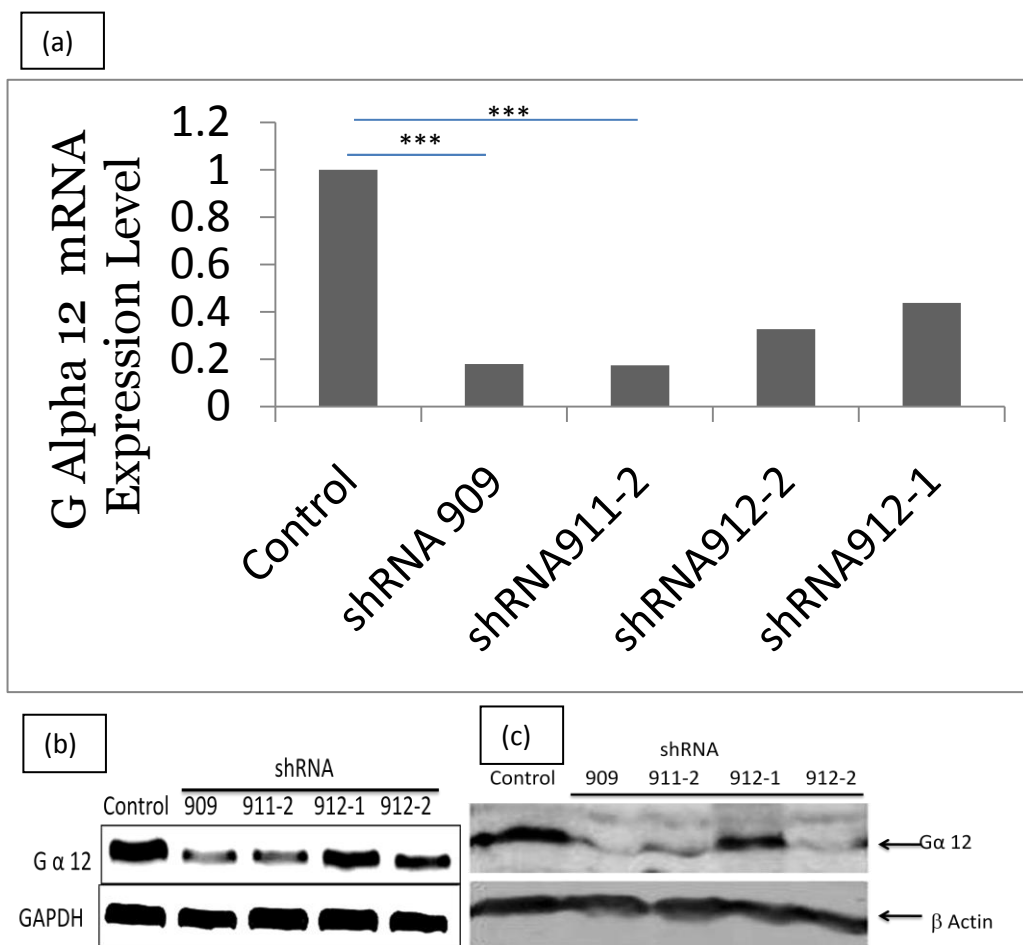


Fig 4. Suppression of G alpha 12 by pre-designed shRNA in PC-3 MM cells. Validation of suppression of G alpha 12 at the mRNA level was done by (a) Quantitative PCR and (b) RT PCR. Suppression of G alpha 12 at protein level was confirmed by (c) Western blot. (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$)

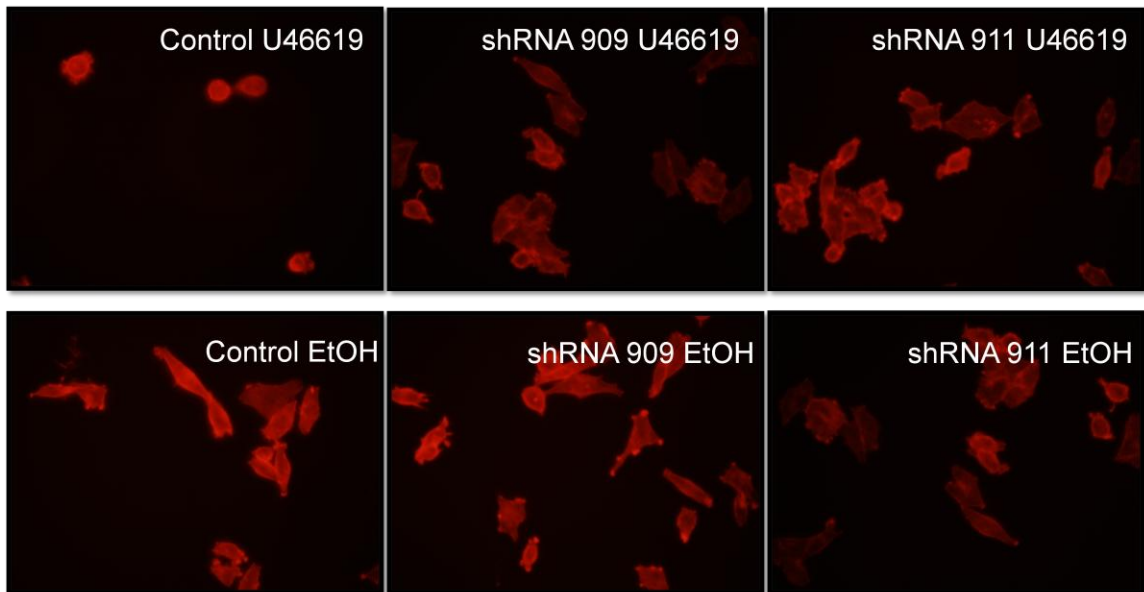


Fig 5. G Alpha 12 depleted cells do not contract when treated with U46619. Inhibition of G alpha 12 decreased cell contraction after activation of thromboxane A2 receptor with U46619. Cells were transfected with short hairpin RNA for G alpha 12, in the presence of a GFP marker construct pEGFP, and then serum starved for 24 hours. Cells were treated with U46619 and EtOH for 15 min. fixed and then stained with TRITC phalloidin staining. Contractions of cells, transfected or untransfected, were scored under an epifluorescence microscope.

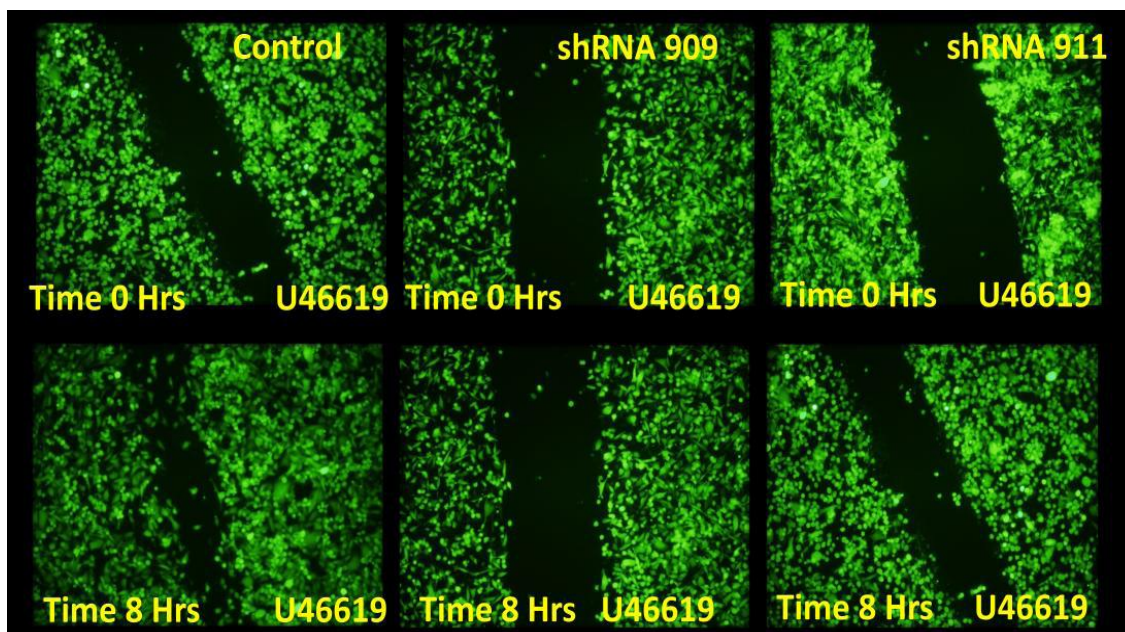


Fig6. Effect of suppression of G alpha 12 on cell motility. Wound healing assay was performed in order to ascertain the effect on cell motility in PC-3 MM cells after silencing G alpha 12. PC-3 MM cells were seeded in a 6 well plate. Cell monolayer was disturbed by producing a scratch when cells reached 90% confluency. After 8 hours, scramble control cells which were treated with U46619 showed increased ability to close the gap in comparison to the cells which had a stable knock down of G alpha 12. Closing of the gap was closely monitored at different time points using an inverted microscope at 200x.

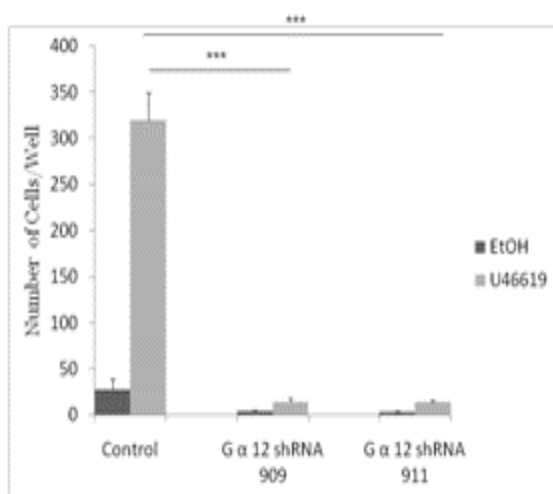
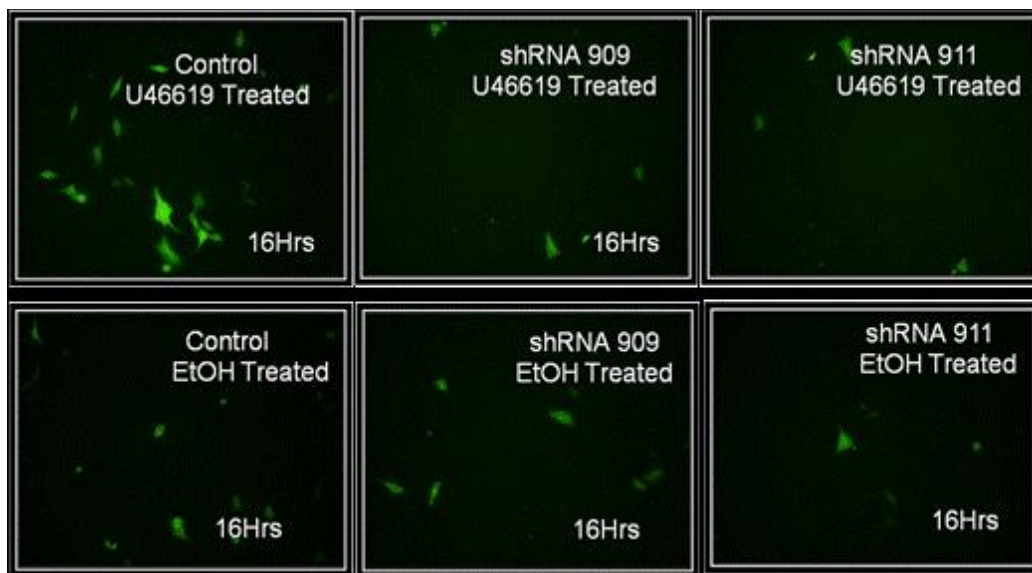


Fig 7. Effect of G alpha 12 suppression on cell migration. Boyden chamber assay was performed to ascertain the effect. Cells are placed in serum free condition in upper chamber and lower chamber contains 10% FBS which acts as a chemo-attractant. Images of migrated cells were taken by inverted microscope at magnification of 200x.

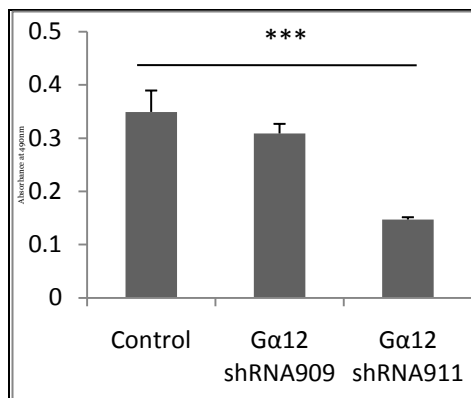
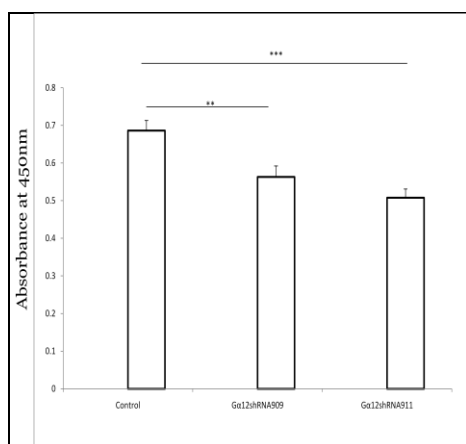
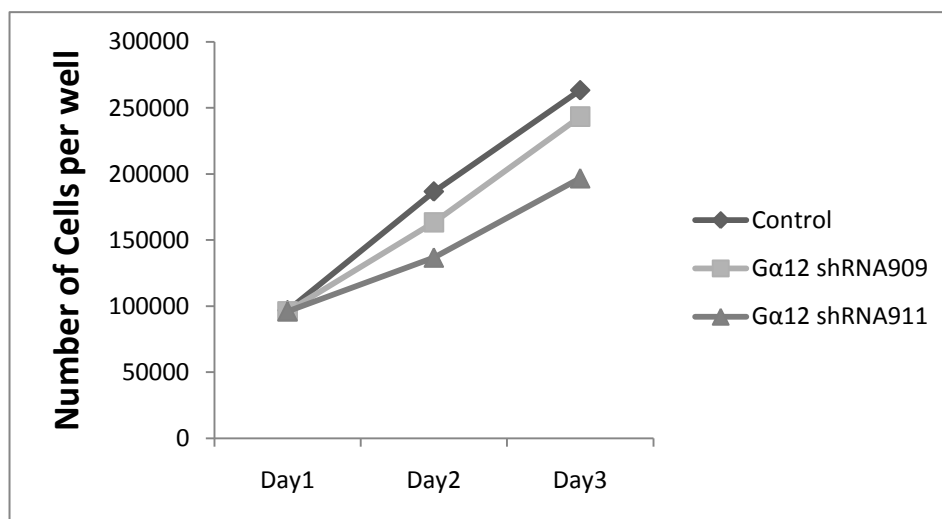


Fig 8. (a) MTS Assay The effect of shRNA on cell proliferation was measured using MTS Assay. PC-3MM cells (10000 cells/well) were seeded in a 96-well plate. The incubated cells were treated with MTS solution after 48 hours. Absorbance was recorded at 490nm.



(b) BrdU Incorporation Assay: 10000 cells/well were plated in 96-well plate and incubated for 24 hours in serum containing medium. After 24 hours, the incubated cells were treated with BrdU solution 100X for 24 hours. Absorbance was recorded at 450nm.



(c) Trypan blue cell proliferation assay 100000 cells/well were plated in 6-well plate and incubated for 24 hours in serum containing medium. The incubated cells were harvested at 24, 48 and 72 hours. The number of cells were counted by Cell Viability Chamber at respective time points.

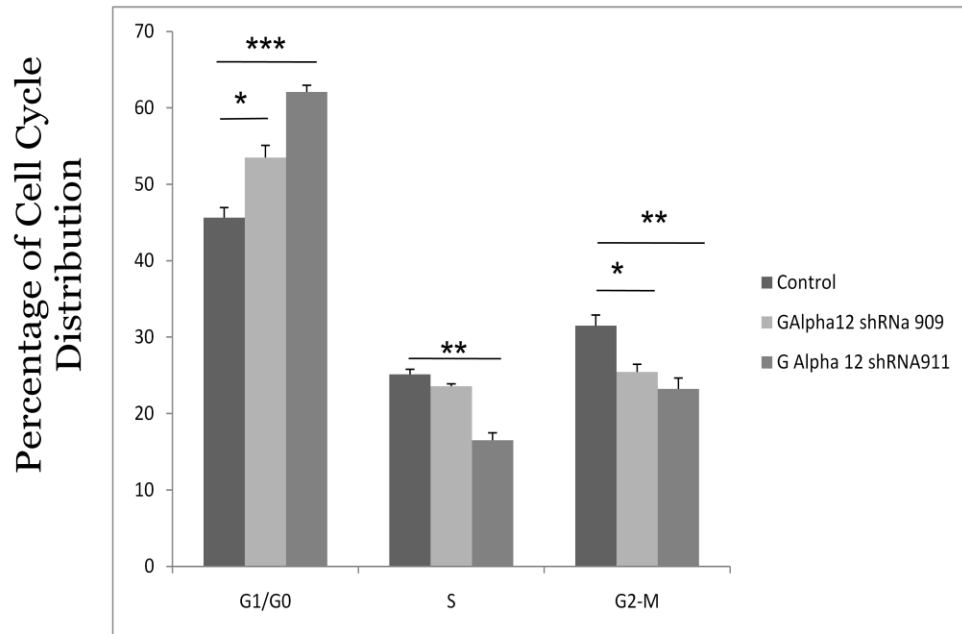


Fig 9. Effect of G alpha 12 silencing on cell cycle distribution. Cell cycle distributions were determined by flow cytometry in PC-3 MM cells at 48 hours post-incubation. Inhibition of G alpha 12 in PC-3MM results in G1/G0 arrest accompanied by concomitant decrease in S and G2-M phase.

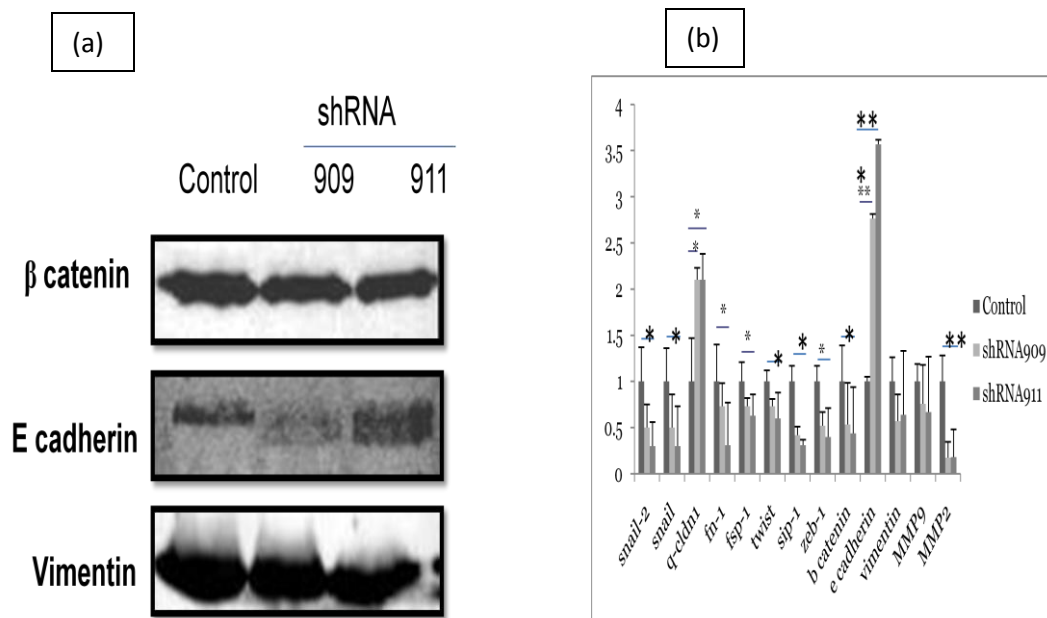


Fig 10. G alpha 12 regulates EMT in PC3 MM cell line. (a) Western blot depicting knock down of G alpha 12 causes up regulation of E cadherin expression along with complimentary decrease in β catenin and vimentin. (b) The mRNA expression level of different EMT associated genes in G alpha 12 depleted cells in PC-3 MM.

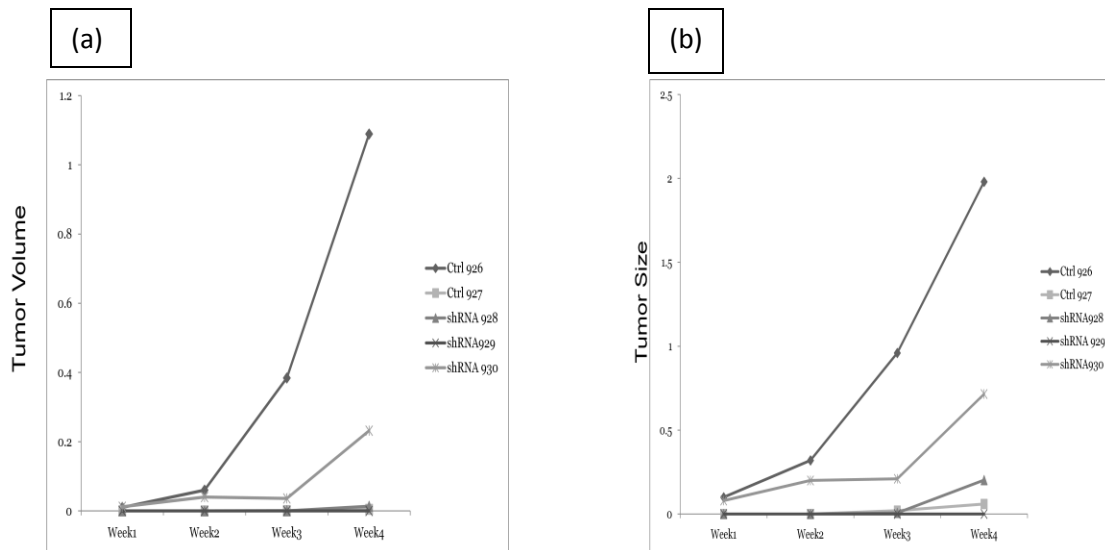


Fig 11. Growth kinetics from our in vivo experiments. (a)Tumor size and (b) Tumor Volume were determined by caliper measurements. Tumors were calibrated for four weeks in two dimension (Length x Width) and tumor volume was calculated by the formula as Length x Width x Width/2

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